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A taxonomic reassessment of the genus *Balsamia* from China

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Abstract

Molecular analysis of the genus *Balsamia* was conducted with ITS and 28S sequences available, including newly gained sequences from Chinese specimens. Combined with the morphological examinations, a new hypogeous species, *Balsamia lishanensis* was described and illustrated from North China, which is morphologically characterized by reddish brown ascomata covered with fine warts, the whitish gleba with numerous small chambers, 3–5 layers peridium with reddish brown polygonal cells and the smooth and regular ellipsoid ascospores with one large oil drop. Two species previously described as *Barssia* were transferred to *Balsamia*. *Balsamia platyspora* was confirmed to be in existence in China based on newly collected specimen. A key to the Chinese *Balsamia* species was provided.

Keywords

Ascomycota, Helvellaceae, Hypogeous fungi, phylogeny, taxonomy

Introduction

The genus *Balsamia* Vittad. (*Helvellaceae*, *Pezizales*), with *B. vulgaris* Vittad. as the type species, was established in the early 19th century (Vittadini 1831), usually forming ectomycorrhizae with both broad leaf and conifer trees (Southworth et al. 2018; Hansen et al. 2019). Geographically, *Balsamia* species are widely distributed across Europe, North America, North Africa and Asia in the temperate regions of the northern hemisphere (Liu and Tao1990; Pegler et al.1993; Southworth et al. 2018; Hansen et al. 2019). Until now, nine *Balsamia* species have been reported from Europe (Vittadini

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1831; Tulasne and Tulasne 1843; Berkely 1844; Tulasne and Tulasne 1851; Schulzer 1870; Petitberghien 1966; Ławrynowicz and Skirgiełło 1984; Kaounas et al. 2015; Hansen et al. 2019), twelve from North America (Southworth et al. 2018), and one from North Africa (Crous et al. 2014; Hansen et al. 2019) In China, this genus is poorly understood as only one species *Balsamia platyspora* Berk. is reported, based on morphological evidence (Liu and Tao 1990).

Recently, two new species of the genus *Barssia* have been described from China (Xu et al. 2018), their taxonomic position, however, needs to be reassessed because Hansen et al. (2019) synonymized *Barssia* under *Balsamia* based on their phylogenetic analysis from three loci (*28S*, *RPB2*, *EF-1α*) and morphological studies. More recently, an un-described *Balsamia* species is recognized when we check the specimens newly collected from north China. In this paper, both the molecular analyses and morphological examinations are conducted for the Chinese samples,, and our aims are:1) to illustrate the position of Chinese *Balsamia* species based on ITS and 28S sequences newly obtained from Chinese *Balsamia* collections with distinct features in this study as well as recently published and used sequences ; 2) to give a detailed characterization of a new species based on morphological features and phylogenetic evidences.

Materials and methods

Morphological studies

Collections were obtained and photographed in the field from Shanxi regions in China, and they were dried and deposited in BJTC (Herbarium, Biology Department, Capital Normal University). One specimen was studied from HMAS (Herbarium Mycologicum Academiae Sinicae, Institute of Microbiology, Chinese Academy of Sciences). Macroscopic characters were recorded from fresh specimens. Microscopic characters were observed in thin sections of dry specimens mounted in 3% KOH, Melzer's reagent (Dring 1971) or 0.1% (w/v) cotton blue in lactic acid. Thirty mature ascospores were measured, and the symbol Q is used to indicate length/width ratios of ascospores in side view.

DNA extraction, PCR amplification and DNA sequencing

Herbarium specimens were crushed by shaking for 30 s at 30 Hz 2–4 times (Mixer Mill MM 301, Retsch, Haan, Germany) in a 1.5 ml tube together with one 3 mm diam. tungsten carbide ball, and total genomic DNA was extracted using the modified CTAB method (Gardes and Bruns 1993). The internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) was amplified using primers ITS1f/ITS4 (White et al. 1990; Gardes and Bruns 1993). The 28S large subunit nrDNA (nrLSU) region was amplified using primers LR0R/LR5 (Vilgalys and Hester 1990). PCRs were

performed in a volume of 50 μl consisted of 4 μl of DNA template; 2 μl of (10 μM) per primer; 25 μl 2× Master Mix (Tiangen Biotech Co., Beijing). The procedure for PCR reaction was: an initial denaturation at 94 °C for 3 min; followed by 35 cycles at 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min; and a final extension at 72 °C for 10 min. The PCR products were sent to Beijing Zhongkexilin Biotechnology Co. Ltd. (Beijing, China) for purifying, sequencing and editing. Validated sequences are stored in the NCBI database (http://www.ncbi.nlm.nih.gov/) under the accession numbers provided (Table1). The other sequences used in the molecular phylogenetic analysis were downloaded from the NCBI database (Suppl. material 1).

Phylogenetic analyses

Two datasets, ITS and 28S, were compiled to identify *Balsamia* species and investigate relationships among species. The taxa *Tuber anniae* and *T. bellisporum* were selected as outgroups. The ITS and 28S sequences were aligned using the MAFFT v.7.110 online program under default parameters (Katoh and Standley 2013), and manually adjusted to allow maximum sequence similarity in Se-Al version.2.03a. (Rambaut 2000). Ambiguously aligned regions and gaps in alignment were excluded by Se-Al version.2.03a. (Rambaut 2000) before the phylogenetic analysis. Alignments were submitted to TreeBASE under accession number S25937. We conducted maximum likelihood (ML), most parsimonious (MP) and Bayesian inference (BI) analyses on the two datasets.

Maximum likelihood (ML) analysis of the dataset was carried out using RAxML 8.0.14 (Stamatakis 2014) and the GTRGAMMA substitution model with parameters unlinked. The ML bootstrap replicates (1000) were computed in RAxML using a rapid bootstrap analysis and search for the best-scoring ML tree. The ML trees were viewed with TreeView32 (Page 2001). Clades with bootstrap support (MLBS) ≥ 70% were considered as significant-supported (Hillis and Bull 1993).

A most parsimonious (MP) analysis was constructed with PAUP* 4.0b10. (Swofford 2002). The bootstrap values were generated using the following settings: 1000 replicate searches on all parsimoniously informative characters using 100 random sequence addition replications and TBR (tree-bisection reconnection) branch swapping

Table 1. Information on newly generated DNA sequences used in this study.

Fungal taxon	Specimen voucher	Locality	ITS	28S
Balsamia lishanensis	BJTC FAN587	Shanxi, China	MT232721	MT232903
	BJTC FAN591	Shanxi, China	MT232899	MT232911
	BJTC FAN676	Shanxi, China	MT232907	MT232902
	BJTC FAN689	Shanxi, China	MT232905	MT232914
	BJTC FAN697	Shanxi, China	MT232908	MT232912
	BJTC FAN714	Shanxi, China	MT232901	MT232913
	BJTC FAN1010	Shanxi, China	MT232900	MT232910
	HMAS 97115	Gansu, China	MT232904	MT232909
Balsamia platyspora	BJTC FAN557	Shanxi, China	MT232906	MT229143

algorithms in PAUP*. Tree statistics (TL), consistency index (CI), retention index (RI) and homoplasy index (HI) were also calculated. Tree was viewed with TreeView32 (Page 2001). Clades with bootstrap support (MPBS) ≥ 70% were considered to be significant (Hillis and Bull 1993).

Bayesian inference (BI) analyses was performed with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) based on the best substitution models determined by MrModeltest 2.3 (Nylander 2004), which were GTR+I+G for the ITS dataset and SYM+I+G for the 28S dataset. Two independent runs of four chains were conducted for 4 000 000 for ITS and 2 000 000 for 28S datasets Markov Chain Monte Carlo generations using the default settings and sampled every 100 generations. The temperature value was lowered to 0.20, burn-in was set to 0.25, and the run was automatically stopped as soon as the average standard deviation of split frequencies reached below 0.01. A 50% majority-rule consensus tree was constructed and visualized with TreeView32 (Page 2001). Clades with Bayesian posterior probability (BPP) ≥ 0.95 were considered as significantly supported (Alfaro et al. 2003).

Results

Phylogenetic analysis

For ITS dataset, we comprehensively collected the ITS sequences of *Balsamia* and the fungi previously described as *Barssia*, and sequences that are high similarity to *Balsamia*. For 28S dataset, we collected all sequences of *Balsamia* and the fungi previously described as *Barssia*, and representative sequence of other genera of *Helvellaceae*. Sequences of each locus were aligned and analyzed separately.

The 28S dataset contained 72 sequences (9 were newly gained in this study), and 4 from the outgroup *Tuber anniae* and *T. bellisporum*. The dataset had an aligned length of 886 characters, of which 578 were constant, 308 were variable, and 278 of these variable sites were informative. The maximum parsimony analysis resulted in one most parsimonious tree with a length (TL) of 842 steps, consistency index (CI) of 0.570, retention index (RI) of 0.896, homoplasy index (HI) of 0.430. MP, ML and BI analyses yielded similar tree topologies, and only the tree inferred from the MP analysis is shown (Fig. 1). The 28S sequences of *Balsamia* were grouped into a distinct clade with high supports (MPBS = 100%, MLBS = 100%, BPP = 1.00). The Chinese materials were well clustered in the *Balsamia* clade (Fig. 1), including the sequences of the fungi previously described as *Barrsia guozigouensis* L. Fan & Y.Y. Xu and *Barssia luyashanensis* L. Fan & Y.Y. Xu (Xu et al. 2018). Three distinct branches with strong supports can be recognized from Chinese collections, respectively representing *Balsamia guozigouensis*, *Balsamia luyashanensis*, and a new species *Balsamia lishanensis* proposed in this study. In addition, the Chinese sequence from BJTC FAN557 grouped together with a reliably identified sequence (MK100252) of *B. platyspora* (Hansen et al. 2019) with strong support value (MPBS = 99%, MLBS = 99%, BPP = 1.00), and they shared 99.83% 28S sequences similarity, indicating the Chinese specimen BJTC FAN557 was *B. platyspora*.

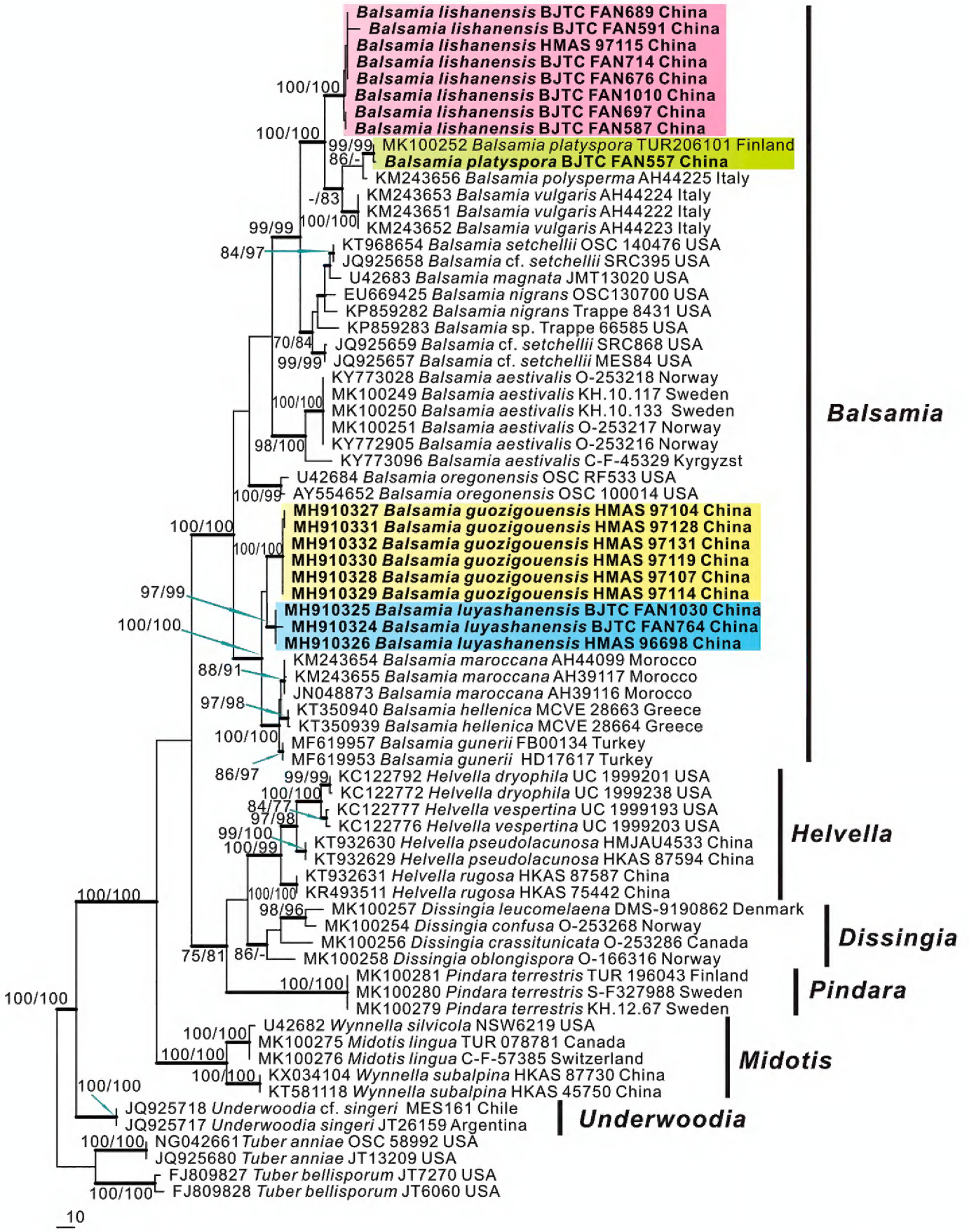
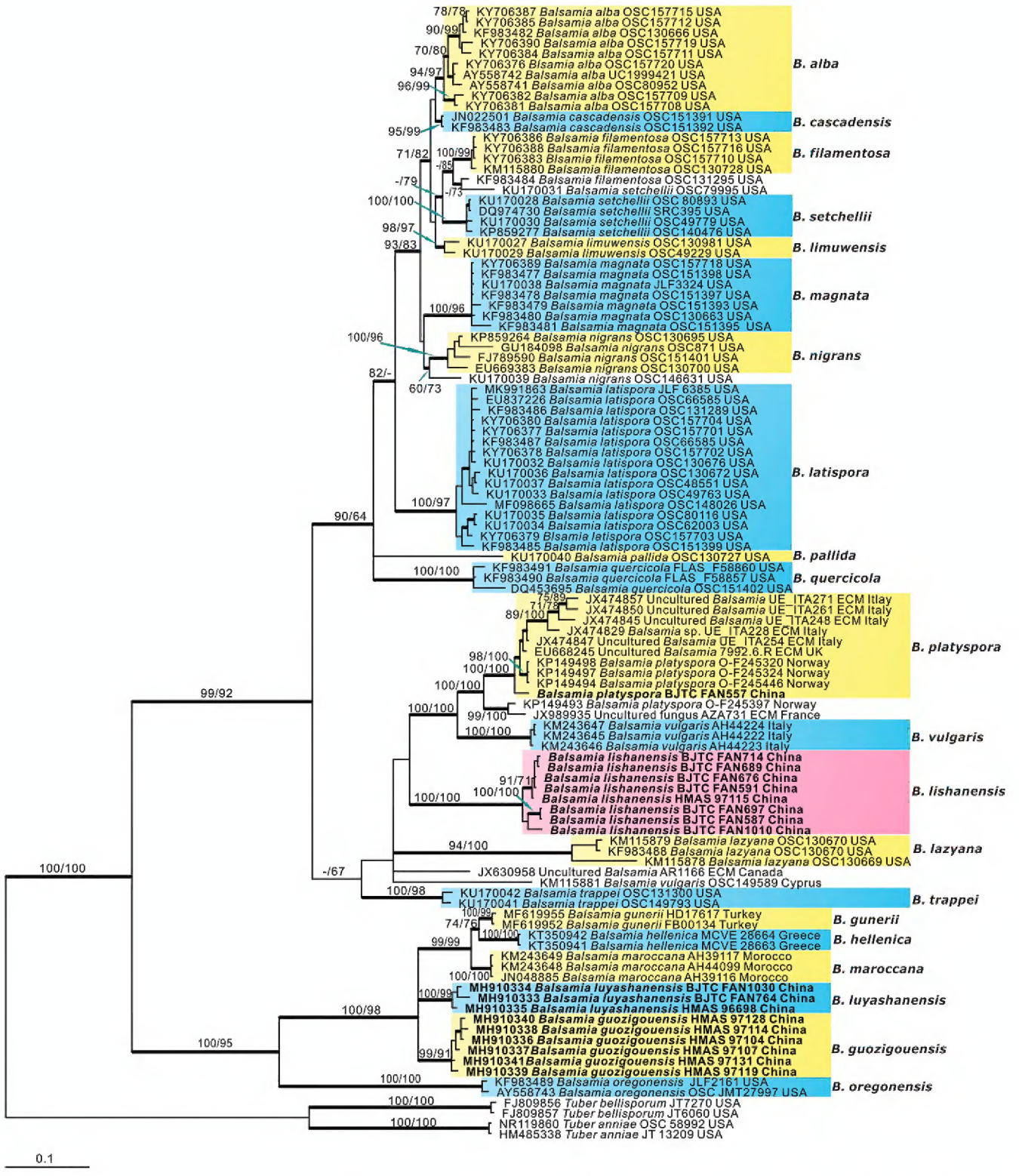

Balsamia lishanensis BJTC FAN689 China
Balsamia lishanensis BJTC FAN591 China
Balsamia lishanensis HMAS 97115 China
Balsamia lishanensis BJTC FAN714 China
Balsamia lishanensis BJTC FAN676 China
Balsamia lishanensis BJTC FAN1010 China
Balsamia lishanensis BJTC FAN697 China
Balsamia lishanensis BJTC FAN587 China
MK100252 *Balsamia platyspora* TUR206101 Finland
Balsamia platyspora BJTC FAN557 China
KM243656 *Balsamia polysperma* AH44225 Italy
KM243653 *Balsamia vulgaris* AH44224 Italy
KM243651 *Balsamia vulgaris* AH44222 Italy
KM243652 *Balsamia vulgaris* AH44223 Italy
KT968654 *Balsamia setchellii* OSC 140476 USA
JQ925658 *Balsamia* cf. *setchellii* SRC395 USA
U42683 *Balsamia magnata* JMT13020 USA
EU669425 *Balsamia nigrans* OSC130700 USA
KP859282 *Balsamia nigrans* Trappe 8431 USA
KP859283 *Balsamia* sp. Trappe 66585 USA
JQ925659 *Balsamia* cf. *setchellii* SRC868 USA
JQ925657 *Balsamia* cf. *setchellii* MES84 USA
KY773028 *Balsamia aestivalis* O-253218 Norway
MK100249 *Balsamia aestivalis* KH.10.117 Sweden
MK100250 *Balsamia aestivalis* KH.10.133 Sweden
MK100251 *Balsamia aestivalis* O-253217 Norway
KY772905 *Balsamia aestivalis* O-253216 Norway
KY773096 *Balsamia aestivalis* C-F-45329 Kyrgyzst
U42684 *Balsamia oregonensis* OSC RF533 USA
AY554652 *Balsamia oregonensis* OSC 100014 USA
MH910327 *Balsamia guozigouensis* HMAS 97104 China
MH910331 *Balsamia guozigouensis* HMAS 97128 China
MH910332 *Balsamia guozigouensis* HMAS 97131 China
MH910330 *Balsamia guozigouensis* HMAS 97119 China
MH910328 *Balsamia guozigouensis* HMAS 97107 China
MH910329 *Balsamia guozigouensis* HMAS 97114 China
MH910325 *Balsamia luyashanensis* BJTC FAN1030 China
MH910324 *Balsamia luyashanensis* BJTC FAN764 China
MH910326 *Balsamia luyashanensis* HMAS 96698 China
KM243654 *Balsamia maroccana* AH44099 Morocco
KM243655 *Balsamia maroccana* AH39117 Morocco
JN048873 *Balsamia maroccana* AH39116 Morocco
KT350940 *Balsamia hellenica* MCVE 28663 Greece
KT350939 *Balsamia hellenica* MCVE 28664 Greece
MF619957 *Balsamia gunerii* FB00134 Turkey
MF619953 *Balsamia gunerii* HD17617 Turkey
KC122792 *Helvella dryophila* UC 1999201 USA
KC122772 *Helvella dryophila* UC 1999238 USA
KC122777 *Helvella vespertina* UC 1999193 USA
KC122776 *Helvella vespertina* UC 1999203 USA
KT932630 *Helvella pseudolacunosa* HMJAU4533 China
KT932629 *Helvella pseudolacunosa* HKAS 87594 China
KT932631 *Helvella rugosa* HKAS 87587 China
KR493511 *Helvella rugosa* HKAS 75442 China
MK100257 *Dissingia leucomelaena* DMS-9190862 Denmark
MK100254 *Dissingia confusa* O-253268 Norway
MK100256 *Dissingia crassitunicata* O-253286 Canada
MK100258 *Dissingia oblongispora* O-166316 Norway
MK100281 *Pindara terrestris* TUR 196043 Finland
MK100280 *Pindara terrestris* S-F327988 Sweden
MK100279 *Pindara terrestris* KH.12.67 Sweden
U42682 *Wynnella silvicola* NSW6219 USA
MK100275 *Midotis lingua* TUR 078781 Canada
MK100276 *Midotis lingua* C-F-57385 Switzerland
KX034104 *Wynnella subalpina* HKAS 87730 China
KT581118 *Wynnella subalpina* HKAS 45750 China
JQ925718 *Underwoodia* cf. *singeri* MES161 Chile
JQ925717 *Underwoodia singeri* JT26159 Argentina
NG042661 *Tuber anniae* OSC 58992 USA
JQ925680 *Tuber anniae* JT13209 USA
FJ809827 *Tuber bellisporum* JT7270 USA
FJ809828 *Tuber bellisporum* JT6060 USA
10
Balsamia
Helvella
Dissingia
Pindara
Midotis
Underwoodia

Figure 1. Phylogenetic tree generated from a maximum parsimonious analysis based on 28S sequences, showing the phylogenetic relationships of *Helvellaceae*. *Tuber anniae* and *T. bellisporum* are the outgroups. Maximum parsimonious bootstrap support values (≥ 70%) and maximum likelihood bootstrap support values (≥ 70%) are indicated above the nodes as MPBS/MLBS. Thick black branches received Bayesian posterior probabilities (BPP) ≥ 0.95. Novel sequences are printed in bold.

The ITS dataset contained 108 sequences (9 were newly gained in this study), and 4 from the outgroup *T. anniae* and *T. bellisporum*. The dataset had an aligned length of 1056 characters, of which 310 were constant, 745 were variable, and 622

of these variable sites were informative. The maximum parsimony analysis resulted in one most parsimonious tree with a length (TL) of 2220 steps, consistency index (CI) of 0.580, retention index (RI) of 0.900, homoplasy index (HI) of 0.420. MP, ML and BI analyses yielded similar tree topologies, and only the tree inferred from the Bayesian analysis is shown (Fig. 2). The ITS sequences of *Balsamia* were grouped into a distinct clade with high supports (MPBS = 100%, MLBS = 100%, BPP = 1.00), and the sequences from the Chinese collection unambiguously clustered in the *Balsamia*


KY706387 Balsamia alba OSC157715 USA
KY706385 Balsamia alba OSC157712 USA
KF983482 Balsamia alba OSC130666 USA
KY706390 Balsamia alba OSC157719 USA
KY706384 Balsamia alba OSC157711 USA
KY706376 Blsamia alba OSC157720 USA
AY558742 Balsamia alba UC1999421 USA
AY558741 Balsamia alba OSC80952 USA
KY706382 Balsamia alba OSC157709 USA
KY706381 Balsamia alba OSC157708 USA
B. alba
JN022501 Balsamia cascadensis OSC151391 USA
KF983483 Balsamia cascadensis OSC151392 USA
B. cascadensis
KY706386 Balsamia filamentosa OSC157713 USA
KY706388 Balsamia filamentosa OSC157716 USA
KY706383 Blsamia filamentosa OSC157710 USA
KM115880 Balsamia filamentosa OSC130728 USA
KF983484 Balsamia filamentosa OSC131295 USA
B. filamentosa
KU170031 Balsamia setchellii OSC79995 USA
KU170028 Balsamia setchellii OSC 80893 USA
DQ974730 Balsamia setchellii SRC395 USA
KU170030 Balsamia setchellii OSC49779 USA
KP859277 Balsamia setchellii OSC140476 USA
B. setchellii
KU170027 Balsamia limuwensis OSC130981 USA
KU170029 Balsamia limuwensis OSC49229 USA
B. limuwensis
KY706389 Balsamia magnata OSC157718 USA
KF983477 Balsamia magnata OSC151398 USA
KU170038 Balsamia magnata JLF3324 USA
KF983478 Balsamia magnata OSC151397 USA
KF983479 Balsamia magnata OSC151393 USA
KF983480 Balsamia magnata OSC130663 USA
KF983481 Balsamia magnata OSC151395 USA
B. magnata
KP859264 Balsamia nigrans OSC130695 USA
GU184098 Balsamia nigrans OSC871 USA
FJ789590 Balsamia nigrans OSC151401 USA
EU669383 Balsamia nigrans OSC130700 USA
KU170039 Balsamia nigrans OSC146631 USA
B. nigrans
MK991863 Balsamia latispora JLF 6385 USA
EU837226 Balsamia latispora OSC66585 USA
KF983486 Balsamia latispora OSC131289 USA
KY706380 Balsamia latispora OSC157704 USA
KY706377 Balsamia latispora OSC157701 USA
KF983487 Balsamia latispora OSC66585 USA
KY706378 Balsamia latispora OSC157702 USA
KU170032 Balsamia latispora OSC130676 USA
KU170036 Balsamia latispora OSC130672 USA
KU170037 Balsamia latispora OSC48551 USA
KU170033 Balsamia latispora OSC49763 USA
MF098665 Balsamia latispora OSC148026 USA
KU170035 Balsamia latispora OSC80116 USA
KU170034 Balsamia latispora OSC62003 USA
KY706379 Blsamia latispora OSC157703 USA
KF983485 Balsamia latispora OSC151399 USA
B. latispora
KU170040 Balsamia pallida OSC130727 USA
B. pallida
KF983491 Balsamia quercicola FLAS_F58860 USA
KF983490 Balsamia quercicola FLAS_F58857 USA
DQ453695 Balsamia quercicola OSC151402 USA
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JX474850 Uncultured Balsamia UE_ITA261 ECM Italy
JX474845 Uncultured Balsamia UE_ITA248 ECM Italy
JX474829 Balsamia sp. UE_ITA228 ECM Italy
JX474847 Uncultured Balsamia UE_ITA254 ECM Italy
EU668245 Uncultured Balsamia 7992.6.R ECM UK
KP149498 Balsamia platyspora O-F245320 Norway
KP149497 Balsamia platyspora O-F245324 Norway
KP149494 Balsamia platyspora O-F245446 Norway
Balsamia platyspora BJTC FAN557 China
KP149493 Balsamia platyspora O-F245397 Norway
JX989935 Uncultured fungus AZA731 ECM France
B. platyspora
KM243647 Balsamia vulgaris AH44224 Italy
KM243645 Balsamia vulgaris AH44222 Italy
KM243646 Balsamia vulgaris AH44223 Italy
B. vulgaris
Balsamia lishanensis BJTC FAN714 China
Balsamia lishanensis BJTC FAN689 China
Balsamia lishanensis BJTC FAN676 China
Balsamia lishanensis BJTC FAN591 China
Balsamia lishanensis HMAS 97115 China
Balsamia lishanensis BJTC FAN697 China
Balsamia lishanensis BJTC FAN587 China
Balsamia lishanensis BJTC FAN1010 China
B. lishanensis
KM115879 Balsamia lazyana OSC130670 USA
KF983488 Balsamia lazyana OSC130670 USA
KM115878 Balsamia lazyana OSC130669 USA
B. lazyana
JX630958 Uncultured Balsamia AR1166 ECM Canada
KM115881 Balsamia vulgaris OSC149589 Cyprus
KU170042 Balsamia trappei OSC131300 USA
KU170041 Balsamia trappei OSC149793 USA
B. trappei
MF619955 Balsamia gunerii HD17617 Turkey
MF619952 Balsamia gunerii FB00134 Turkey
B. gunerii
KT350942 Balsamia hellenica MCVE 28664 Greece
KT350941 Balsamia hellenica MCVE 28663 Greece
B. hellenica
KM243649 Balsamia maroccana AH39117 Morocco
KM243648 Balsamia maroccana AH44099 Morocco
JN048885 Balsamia maroccana AH39116 Morocco
B. maroccana
MH910334 Balsamia luyashanensis BJTC FAN1030 China
MH910333 Balsamia luyashanensis BJTC FAN764 China
MH910335 Balsamia luyashanensis HMAS 96698 China
B. luyashanensis
MH910340 Balsamia guozigouensis HMAS 97128 China
MH910338 Balsamia guozigouensis HMAS 97114 China
MH910336 Balsamia guozigouensis HMAS 97104 China
MH910337Balsamia guozigouensis HMAS 97107 China
MH910341Balsamia guozigouensis HMAS 97131 China
MH910339 Balsamia guozigouensis HMAS 97119 China
B. guozigouensis
KF983489 Balsamia oregonensis JLF2161 USA
AY558743 Balsamia oregonensis OSC JMT27997 USA
B. oregonensis
FJ809856 Tuber bellisporum JT7270 USA
FJ809857 Tuber bellisporum JT6060 USA
NR119860 Tuber anniae OSC 58992 USA
HM485338 Tuber anniae JT 13209 USA
0.1

Figure 2. Phylogenetic tree generated from Bayesian analysis based on ITS sequences, showing the phylogenetic relationships of *Balsamia*. *Tuber anniae* and *T. bellisporum* are the outgroups. Maximum parsimonious bootstrap support values (≥ 70%) and maximum likelihood bootstrap support values (≥ 70%) are indicated above the nodes as MPBS/MLBS. Thick black branches received Bayesian posterior probabilities (BPP) ≥ 0.95. Novel sequences are printed in bold.

clade, including the sequences of the fungi previously described as *Barrsia guozigouensis* and *Barssia luyashanensis* (Xu et al. 2018) (Fig. 2). The sequences of all Chinese collections excepting specimen BJTC FAN557 were grouped into three independent clades with strong supports (Fig. 2), respectively representing *Balsamia guozigouensis*, *Balsamia luyashanensis* and a new species *Balsamia lishanensis* proposed in this study. The sequence of BJTC FAN557, which was identified as *B. platyspora* by morphology and 28S phylogeny (Fig. 1) in this study, formed a strong support clade together with nine European sequences isolated from ascomata of *Balsamia platyspora* or ectomycorrhizal root tips of *Balsamia*. These ten samples showed high sequences similarity so the clade was considered as representing *B. platyspora*.

Based on the above phylogenetic analyses (Figs 1, 2), we concluded that *Barrsia guozigouensis* and *Barssia luyashanensis* should be transferred to *Balsamia*. The clade of *B. lishanensis* was a distinct species and represented a new species. The specimen BJTC FAN557 should be recognized as the European *Balsamia platyspora*.

Taxonomy

Balsamia lishanensis **L. Fan & Y.Y. Xu, sp. nov.**
MycoBank No: 834962
Figure 3

Etymology. *lishanensis*, Lishan Mountain, referring to the locality where the type specimen was collected.

Holotype. China. Shanxi Province, Yuanqu County, Lishan Mountain Shunwangping Scenic Area, alt. 2300 m, 17 October 2016, in soil under *Pinus armandii* Franch., M. Chen CM019 (BJTC FAN676).

Ascomata subglobose to irregularly subglobose, 3–14 × 2–12 mm in fresh, reddish brown when fresh, usually with some superficial furrows, surface covered with verrucose or fine warts, warts obtuse or pointed, 270–400 μm wide and 150–300 μm high. Odor light, mushroom flavor. Gleba solid, white to cream white, with numerous irregular canals and chambers of around 1 mm width. Peridium 150–350 μm thick, two-layered, outer layer pseudoparenchymatous, 90–190 μm thick, composed of 3–5 layers of reddish brown polygonal cells with 4-6 sides, cells 15–35 × 10–27 μm, walls 4.0–8.0 μm thick, the outermost cells reddish-brown, and gradually light-yellow to hyaline towards inner side; inner layer 60–150 μm thick, composed of interwoven hyphae, that is more or less parallel to the surface of peridium, hyphae hyaline, 2.5–6.0 μm wide. Paraphyses line the surface of chamber, arranged like a fence, 3–4 × 50 μm, but disorganized in the mature ascomata, usually not well-defined. Asci 8-spored, hyaline, citriform or fusiform, 55–80 × 27–38 μm (not including stalk), inamyloid, with a slender-stalk of 13.5–35 × 5–10 μm, spores irregularly arranged in ascus. Ascospores ellipsoid, smooth, hyaline, inamyloid, 20.6–25.6 × 12.9–15 μm (av. 23.5 × 14.0 μm), Q (L/I) = 1.55–1.80 (Qm = 1.68) (n = 30), usually containing one large oil drop and several small droplets.

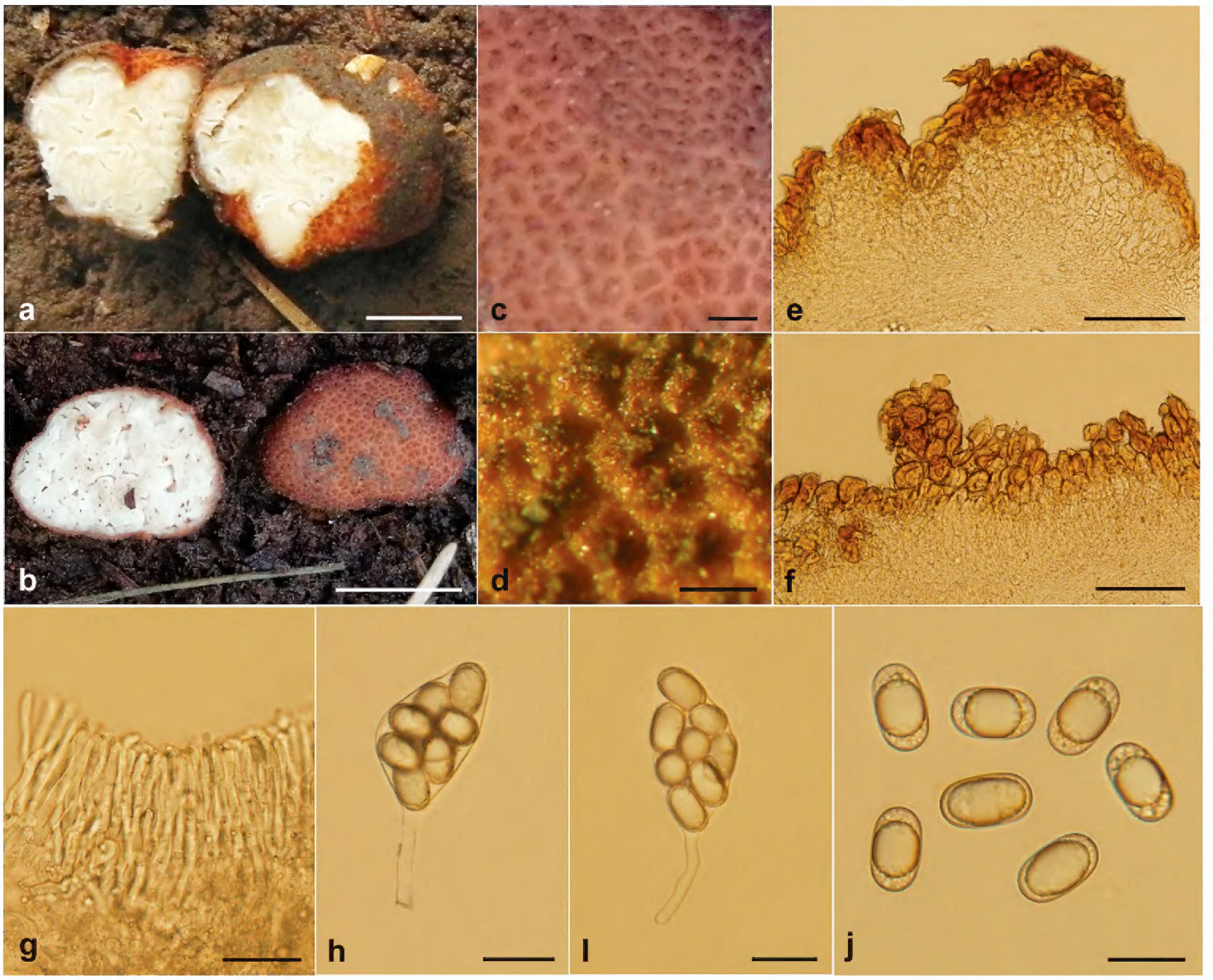

Figure 3. *Balsamia lishanensis* (BJTC FAN676, holotype) **a, b** ascomata **c** warts (when fresh) **d** warts (when dry) **e, f** peridium **g** paraphyses **h, i** mature ascus **j** ascospores. Scale bars: 5 mm (**a, b**); 500 μm (**c**); 300 μm (**d**); 100 μm (**e, f**); 25 μm (**g, h, i**); 20 μm (**j**).

Other materials examined. China. Shanxi Province, Yuanqu County, Lishan Mountain Shunwangping Scenic Area, alt. 2300m, 16 August 2016, in soil under *Pinus armandii* Franch., K.B. Huang HKB003 (BJTC FAN587); *ibid.*, 16 August 2016, in soil under *Pinus armandii* Franch., B.D. He HBD014 (BJTC FAN 591); *ibid.*, 17 October 2016, in soil under *Pinus armandii* Franch., K.B. Huang HKB039 (BJTC FAN689); *ibid.*, 17 October 2016, in soil under *Pinus armandii* Franch., X.Y. Sang SXY015 (BJTC FAN697); *ibid.*, 17 October 2016, in soil under *Pinus armandii* Franch., K.B. Huang HKB031 (BJTC FAN714); China. Shanxi Province, Ningwu County, Xiaoshidong Village, Guancen Mountain, alt. 2000m, 12 October 2017, in soil under *Picea asperata* Mast., L.J. Guo GLJ001 (BJTC FAN1010); China. Gansu Province, Bailongjiang Forestry Bureau, Seventh Forest Farm, alt. 2500m, 14 July 2002, in soil under *Pinus* sp., D.J. Ren & M.S. Song 02-034 (HMAS 97115).

Notes. *Balsamia lishanensis* was diagnosed by the combination of reddish brown ascomata covered with fine warts, the whitish gleba with numerous small chambers open to 1 mm, 3–5 layers peridium reddish brown polygonal cells and the smooth and regular ellipsoid ascospores with one large oil drop. There are four *Balsamia* spe-

cies similar to *B. lishanensis* in morphology. Of them, *B. vulgaris* differed by its large ascospores of (23–) 26–32 (–36) × 11.5–14 (–16) μm, *Balsamia lazyana* and *B. trappei* by their narrow ascospores, which are 19.5–27 × 8–11.5 μm in *B. lazyana* and 24–26 × 11.5–13.5 μm in *B. trappei*, *B. platyspora* by its short-ellipsoid ascospores of 19–22–28 × 12–13–16 μm (ca. 20 × 13 μm). Phylogenetic analysis revealed that the sequences of *B. lishanensis* were grouped into an independent clade with strong support value (Figs 1, 2). DNA analysis showed that *B. lishanensis* shared less than 87.19% identity in ITS sequence with other *Balsamia* species. These supported the erection of the new species.

Balsamia platyspora **Berk. Ann. Mag. Nat. Hist.13: 358(1884)**
Figure 4

Materials examined. China. Shanxi Province, Yuanqu County, Lishan Mountain Shunwangping Scenic Area, alt. 2200m, 16 August 2016, in soil under *Pinus armandii* Franch., Y.W. Wang WYW012 (BJTC FAN557).

Notes. *Balsamia platyspora* is distributed in Europe, North America and Asia (Berkely 1844; Gilkey 1939). In China, it is reported as early as 1990 from Shanxi Province based on morphological evidences (Liu and Tao 1990), but unfortunately, we have been unable to find the voucher specimen. In this study, our molecular analysis based on 28S sequences (Fig. 1) and ITS sequences (Fig. 2), and morphological studies confirmed the occurrence of this species in China based on the new collections from the Shanxi Province where this species was harvested originally by Liu and Tao (1990). *Balsamia platyspora* is mainly characterized by its minor subglobose ascomata,

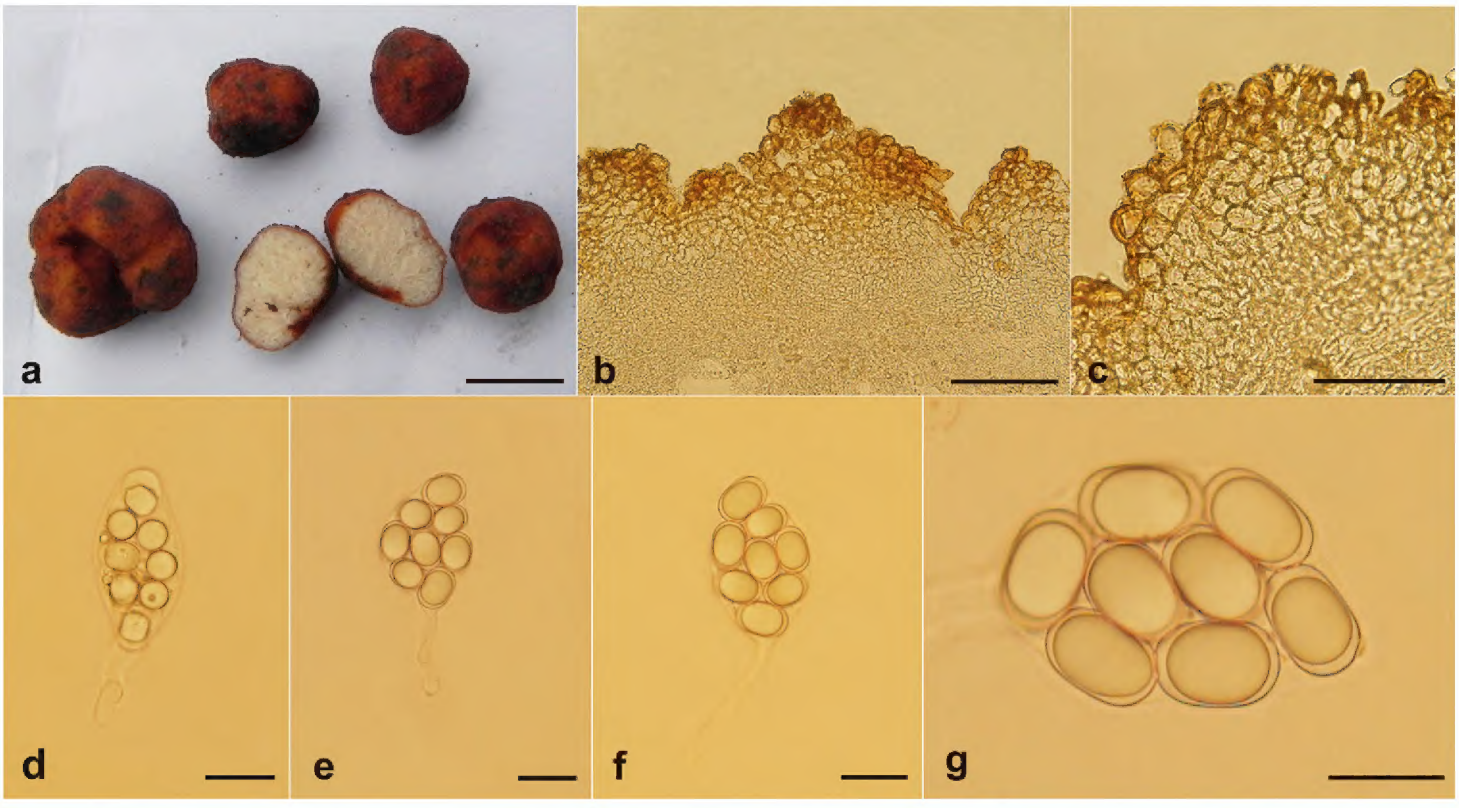

Figure 4. *Balsamia platyspora* (BJTC FAN557) **a** ascomata **b** peridium **c** warts **d** immature ascus **e, f** mature ascus **g** ascospores. Scale bars: 1 cm (**a**); 100 μm (**b, c**); 25 μm (**d, e, f**); 20 μm (**g**).

reddish brown to dark brown warts, white to yellowish white gleba with around 1 mm chambers, citriform or broadly elliptic asci, shortly elliptic ascospores 19–22–28 × 12–13–16 µm (ca. 20 × 13 µm) (Berkeley 1844; Hawker 1954; Pegler et al.1993), our specimen well matched the characteristics.

***Balsamia guozigouensis* (L. Fan & Y.Y. Xu) L. Fan & Y.Y. Xu., comb. nov.**
MycoBank No: 834963

Basionym. *Barssia guozigouensis* L. Fan & Y.Y. Xu, Phytotaxa 374(2): 135 (2018).

Holotype. China. Xinjiang Autonomous Region, Huocheng County, Guozigou Forest Park, alt. 1800m, in soil under *Picea schrenkiana* Fisch. & C.A. Mey., 11 August 2003, W.P. Wu & M. S. Song 060 (HMAS 97107).

Illustrations – Xu et al. (2018: Fig. 4)

Notes. This species is recently described from Xinjiang Autonomous Region, China, under *Picea schrenkiana* Fisch. & C.A. Mey. So far it is known only from the type locality. *Balsamia guozigouensis* can be recognized by its distinctly warty ascomata, solid gleba with small and irregular chamber and irregularly clavate asci. Phylogenetically, it was closely related to *B. luyashanensis* (Fig. 2), but the latter differs in its ascomata with fine warts and gleba without chambers (Xu et al. 2018).

***Balsamia luyashanensis* (L. Fan & Y.Y. Xu) L. Fan & Y.Y. Xu., comb. nov.**
MycoBank No: 834964

Basionym. *Barssia luyashanensis* L. Fan & Y.Y. Xu, Phytotaxa 374(2): 134 (2018).

Holotype. China. Shanxi Province, Ningwu County, Qiuqiangou Village, Luyashan Mountain, alt. 2100m, 25 August 2017, in soil under *Picea* sp., M. Chen CM023 (BJTC FAN764).

Illustrations – Xu et al. (2018: fig. 3)

Notes. *Balsamia luyashanensis* is also recently described from the Luyashan Mountain of Shanxi Province, China, under *Picea* sp. So far it is known only from the type locality. The species can be recognized by its red brown ascomata with fine warts, gleba without chambers and irregularly clavate asci (Xu et al. 2018). The species was similar in appearance of ascomata to *B. gunerii* and *B. hellenica* but *B. gunerii* can be separated by its subglobose to ovoid ascospores and gleba with irregularly sinuous, labyrinth-like veins (Doğan et al. 2018; Hansen et al. 2019), while *B. hellenica* by its ovoid ascospores (Kaounas et al. 2015; Hansen et al. 2019).

Key to Chinese species of *Balsamia*

1 Ascomata with an obvious apical depression **2**
– Ascomata without an obvious apical depression **3**

2	Surface with distinct warts, solid gleba scattered with some small and irregular chambers ..... ***B. guozigouensis***
–	Surface with fine warts, solid gleba without chambers..... ***B. luyashanensis***
3	Ascospores long-ellipsoid, (20.6–25.6 × 12.9–15 μm, Q = 1.55–1.80) ..... ***B. lishanensis***
–	Ascospores short-ellipsoid, 19–22–28 × 12–13–16 μm (ca. 20 × 13 μm) (Hawker 1954) ..... ***B. platyspora***

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Supplementary material I

Table S1. Taxa used in this study and their GenBank accession numbers for ITS and 28S sequence data

Authors: Yu-Yan Xu, Xiang-Yuan Yan, Ting Li, Li Fan

Data type: Microsoft Word Document (.docx)

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